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(71) Applicants (for all designated States except US): THE UNIVERSITY OF MELBOURNE [AU/AU]; Grattan Street, Parkville, VIC 3052 (AU). MEAT RESEARCH CORPORATION [AU/AU]; Aetna Life Tower, Hyde Park Square, Cnr Elizabeth and Bathurst Streets, Sydney, NSW 2000 (AU).			
(72) Inventors; and (75) Inventors/Applicants (for US only): MEEUSEN, Elza, Nicole, Theresia [BE/AU]; 17 Wolseley Parade, Kensington, VIC 3550 (AU). WALKER, John [AU/AU]; 26 Clapham Street, Balwyn, VIC 3103 (AU). ASHMAN, Keith [AU/AU]; 10 Arawata Parade, Melton, VIC 3337 (AU). NEWTON, Susan, Elizabeth [AU/AU]; 3 Lebanon Street, Strathmore, VIC 3041 (AU).			
(74) Agent: PHILLIPS ORMONDE & FITZPATRICK; 367 Collins Street, Melbourne, VIC 3000 (AU).			
(54) Title: PROTECTIVE ANTIGENS AGAINST PARASITES			
(57) Abstract A putative protective antigen or fragment thereof against infections selected from the group consisting of <i>Ostertagia circumcincta</i> , <i>Trichostrongylus colubriformis</i> and <i>Fasciola hepatica</i> or related infections, being antigens selected from antigens having approximate molecular weights in the region of 26-36 and 91-105 kilodaltons, an antigen having an approximate molecular weight of 32-35 kilodaltons, and antigens having approximate molecular weights in the region of 28 kilodaltons, 32 kilodaltons, 37 kilodaltons, 42 to 100 kilodaltons, 54 to 55 kilodaltons and > 200 kilodaltons, respectively.			

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After hatching, larval development to the third stage (L3) occurs on the pasture. L3 larvae are ingested by grazing sheep and undergo further development in the abomasum. The L4 stage develops in the abomasal crypts; development may be slowed or arrested depending on the immune status of the sheep and on the season. Larvae may lie dormant in the mucosal crypts over winter before developing into mature egg producing adults in the spring. The sudden synchronised rise in parasite numbers can cause significant morbidity. Tissue damage due to feeding of late larval stages and adults on mucosa causes serum leakage and hypertrophy of the abomasal lining with subsequent interference with abomasal function and consequently poor growth of the animal. A similar pathology is caused in cattle by the related parasite O. ostertagii.

Despite the importance of Ostertagia species in causing considerable economic losses in the sheep and cattle industry in Australia and overseas, no successful vaccine has been developed in the prior art against this parasite.

Fasciola hepatica (liverfluke) is a parasite belonging to the Trematode family, which can infect a variety of wild and domestic animal species and is of particular economic importance to the sheep and cattle industry. Among the species studied, rats are the only host that can develop a strong, immunologically based immunity to reinfection (reviewed in Haroun, E T M and G V Hillyer, Vet Parasitol 20 63-93, 1986). Antigens strongly recognised by the rat immune system are therefore of particular importance to vaccination strategies and are described in the present application. Australian Patent 640364 describes a protective antigen against Fasciola hepatica infections, having an approximate molecular weight of 120 to 125 kilodaltons. This antigen was differentially recognised between cattle and sheep and is completely different from the antigens described in the present application.

Infections with Trichostrongylus spp. occur in the first 3-4 metres of small intestine in sheep and cause reduced wool production and body growth, illthrift and scouring. Death can result in severe infections. It is also an economically significant disease but no successful vaccine has been developed in the prior art against this parasite.

It is accordingly an object of the present invention to overcome, or at least alleviate, one or more of the difficulties and deficiencies related to the prior art.

Accordingly, in a first aspect, the present invention provides a putative protective antigen, or fragment thereof, against Ostertagia circumcincta, or related infections, selected from antigens having approximate molecular weights in the region of 26-36 and 95-105 kilodaltons, as hereinafter described. The antigens may also be present in other species and strains of parasites.

The 26-36 kD O. circumcincta antigenic region may include a doublet antigen in the 32-36kD position. The doublet antigen may be a lectin-like β -galactoside-binding protein. The 32-36 kD doublet antigen may include one or more of the peptide sequences

- 1) SAHGPPGQ
- 2) FPHGPSYQHGYA
- 3) IVTHPNR

The lower bands in the 26-36 kD O. circumcincta antigenic region contain proteins homologous to tropomyosin and glutathionine S-transferase.

The O. circumcincta sequences homologous to

(A) tropomyosin are:

1. N-terminal sequence: MKAEVVRQALK
2. Internal sequence : VEADLERAEERAEAAAGENKVVVL

(B) Glutathionine S-transferase:

N-terminal: VQYKLYYFDGRXAAEV

In a further preferred aspect of the present invention there is provided a putative protective antigen, or fragment thereof, against Trichostrongylus colubriformis, or related infections, having an approximate molecular weight of 32-35 kilodaltons, as hereinafter described. The protective antigen may be a doublet.

The T. colubriformis antigen is in the same position on SDS-PAGE as the O. circumcincta doublet antigen referred to above and it is therefore possible that these are essentially similar molecules but with species-specific epitopes recognised most strongly by the homologous supernatant antibody probes.

In a still further aspect of the present invention there is provided a putative protective antigen, or fragment thereof, against Fasciola hepatica, or related infections, selected from antigens having approximate molecular weights in the region of 28 kilodaltons, 32 kilodaltons, 37 kilodaltons, 42 to 100

kilodaltons, 54 to 55 kilodaltons and >200 kilodaltons, as hereinbefore described. Similar antigens may be present in other Fasciola species, for example Fasciola gigantica and in other trematode parasites for example Schistosoma spp.

The >200 kilodalton F. hepatica antigen may be a doublet antigen. The
5 F. hepatica antigens are uniquely recognised by supernatant from mesenteric lymph node (MLN) cells in immune, challenged rats.

The 32 kD antigen may include an N-terminal peptide sequence KPNYKRQFEPFSDELIHYINLE.

The 54-55 kD antigen may include an N-terminal peptide sequence
10 LEDNGRTHWAVLVA.

It will be understood that recombinant protein antigens may be used in place of the native antigens extracted from the parasites.

Fragments of the antigen(s) containing protective epitopes, and synthetic peptides containing protective epitopes may be substituted for the entire native or
15 recombinant molecule(s) in both vaccines and diagnostic tests.

The antigen(s) may be present in other species of parasites and would thus be of use in vaccination and diagnosis of other diseases besides that caused by the designated pathogens.

It will be further understood that antibody raised against these antigens
20 may be used in diagnostic tests or as an immunoprophylactic agent, whether polyclonal or monoclonal.

The protective antigens according to the present invention may be produced utilising the method as described in Australian Patent 640364, referred to above. Accordingly, in a further aspect, the present invention provides a
25 method for preparing an antigen associated with a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species, as described above, which method includes

providing

a sample of a disease pathogen selected from Fasciola, Ostertagia
30 and Trichostrongylus species, and related species; and

a corresponding antibody probe including at least one antibody against the respective disease pathogen produced by a method including

providing a biological sample from an immune animal taken a short time after the immune animal has been challenged with a pathogen or pathogen extract selected from Fasciola, Ostertagia and Trichostrongylus species, and related species;

5 isolating cells from the biological sample;

culturing cells in vitro in a suitable culture medium; and

harvesting antibodies produced from said cells,

probing the pathogen sample to detect at least one antigen with the corresponding antibody probe; and

10 isolating the antigen detected.

The disease pathogen sample is preferably a parasite, parasite extract or parasitic section thereof. The Ostertagia pathogen may be Ostertagia circumcincta or Ostertagia ostertagia. The Trichostrongylus pathogen may be a Trichostrongylus colubriformis or Trichostrongylus axei. The Fasciola species
15 may be Fasciola hepatica or Fasciola gigantica.

In a preferred aspect, the sample of the disease pathogen may be taken at a stage of development during which it is thought to be most susceptible to attack.

It is postulated that the time at which the disease pathogen sample is
20 taken is important since a parasite is vulnerable for only a short time after entering the subject after which it may change structure and is no longer vulnerable to immune attack and may no longer express protective antigens.

For example, in the cases of the disease pathogens, F. hepatica, O. circumcincta and I. colubriformis, it may be suitable to take the sample from the
25 larval stage.

The animal from which the biological sample may be taken may be of any suitable type. The animal from which the biological sample is taken may be an immune animal. The biological sample may be taken a short time after the immune animal has been challenged with a pathogenic infection. The animal
30 may be an animal such as sheep or cattle.

The biological animal sample may be of any suitable type. The biological sample may be from animal tissues, organs, blood, lymph or lymph nodes. The biological sample may be taken from any section of the infected animal.

However, it is preferred that the samples be taken from the infected site or an area of a lesion which may be formed in certain diseases or an area close to or draining from the infected site or a lesion such as in the lymph nodes. Preferably samples may be taken from the hepatic lymph node, abomasal lymph node or mesenteric lymph node. Serum/plasma samples are not preferred as the biological samples. It has been found that the majority of antibodies found in a serum/plasma sample are irrelevant to protection or specific diagnosis of a pathogen or are unrelated to the pathogen.

In contrast, the probes used in the present invention are highly enriched in pathogen-specific antibodies and can be selected to be restricted to the pathogen-stage of particular importance to protective immunity.

The cells isolated from the biological sample may include B cells. The cells may be isolated similarly at a time known to include a secretion and/or antibody producing period. Alternatively, the cells may include memory cells which may be generated at a later stage in certain diseases.

Thus, preferably the cells are taken a short time after in vivo stimulation, preferably within approximately 2 to 13 days thereafter with, for example, the relevant parasite stage thereby resulting in the in vivo induction of antibody forming cells which will secrete specific antibodies into the culture medium after in vitro incubation. No, or very few antibodies may be secreted in culture medium without prior in vivo stimulation of resting lymphocytes.

In vitro secretion of antibodies in the culture medium by recently activated B cells may be enhanced by the addition of helper factors to the cultures. The helper factors may be cytokines used alone or in combination, including interleukin 1, 2, 3, 4, 5, 6, 7 and 8, colony stimulating factors, interferons and any other factors that may be shown to have an enhancing effect on specific antibody secretion by B cells.

The method of producing the antibody probe may include a further step of activating the cells isolated to proliferate and secrete and/or release antibodies.

The cell activation step may include adding a cell activating agent to the culture medium. The cell activating agent may be parasite-derived or may be selected from mitogens and helper factors produced by leucocytes, or their synthetic equivalents or combinations thereof.

The mitogens may be selected from products derived from pokeweed (*Phytolacca americana*) also known as pokeweed mitogen (PWM), phorbolmyristic acid (PMA), polyvinyl-pyrrolidone (PVP), polyadenylic-polyuridylic acid (poly(A-U)), purified protein derivate (PPD), polyinosinic-polycytidilic acid (poly(I-C)), lipopolysaccharide (LPS), staphylococcal organisms or products thereof, Bacto- streptolysin O reagent (SLO), Staphylococcal phage lysate (SPL), Epstein-Barr virus (EBV), Nocardia water-soluble mitogen (NWEM), phytohemagglutinin (PHA) Concanavalin A (Con A) and dextran-sulphate and mixtures thereof. The cell proliferation agent may be any agent that indirectly or directly results in B cell proliferation and/or antibody secretion such as solid-phase anti-immunoglobulin. The helper factors may be cytokines including interleukin 1, 2, 3, 4, 5, 6, 7 and 8, colony stimulating factors, interferons and any other helper factors that may be shown when added alone, or in combination with other factors and agents to have an enhancing effect on specific B cell proliferation and/or antibody secretion. This in no way is meant to be an exhaustive list of mitogens and cell activating agents including helper factors.

The in vitro culturing of the cells may be conducted with or without prior steps to separate sub-populations of cells. The harvesting of antibodies may be conducted by harvesting of the supernatant from the culture medium. This supernatant contains antibodies secreted by these cells during the in vitro culture or artificially released from the B cells, for example by lysis of the B cells. It has been found, surprisingly, that the antibody-containing supernatants may be used directly to detect antigens of a pathogen.

In a preferred aspect, the sample of disease pathogen may be mixed with a standard buffer solution and placed on a standard support such as an SDS-polyacrylamide gel to separate the proteins contained therein. The separated proteins may then be transferred to nitro-cellulose, nylon or other sheets.

The corresponding antibody probe produced as described above may be utilised simply in the form of the supernatant harvested from the culture medium. Alternatively, the antibodies may be separated and purified.

The antibody contained in the culture medium may be used for purification of the antigen. An affinity purification, preferably immuno-affinity purification may be used.

The antigen located as described above may be detected utilising any suitable assay technique.

The antibody probing step may accordingly further include subjecting the product produced thereby to a detection assay.

5 The detection assay may include Western blot techniques. The detection assay may be an immunoprecipitation assay, a radioimmunoassay, an enzyme-linked immunoassay or immunofluorescent assay.

Accordingly, the antigens may be purified by a method which includes providing

10 a crude antigen mixture;

 an antibody against a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus, and related species, immobilised on a suitable support;

 subjecting the crude antigen mixture to an affinity chromatography

15 utilising the immobilised antibody; and

 isolating the purified antigen so formed.

The antibody can be obtained from the culture supernatant probe described above by conventional methods. For example methods usually used to purify immunoglobulins from serum or plasma, e.g. precipitation with ammonium sulphate, fractionation with caprylic acid, ion exchange chromatography or by binding and elution from immobilised protein G or protein A may be utilised.

Antibody so obtained can then be coupled to suitable supports, e.g. CNBr-activated Sepharose 4B (Pharmacia) Affi-gel (Bio-Rad) or other affinity chromatography supports able to bind proteins.

25 Immobilised antibody can then be applied to the fractionation and purification of specific antigen from a complex parasite extract by affinity chromatography. After binding of antigen to immobilised antibody, unbound macromolecular species can be washed away from the solid support with, e.g. buffers containing 1.5M NaCl. Subsequently the antigen can be eluted from the affinity column with, e.g. low or high pH buffer or buffers containing chaotropic ions, e.g. 0.5 - 3.0 M sodium thiocyanate.

30 The antigens isolated or located may be used in the preparation of monoclonal antibodies.

Accordingly, the present invention further provides a method for producing a monoclonal antibody against an antigen of a disease pathogen as described above, which method includes

providing

5 a B cell capable of producing antibodies against said protective antigen, or fragments thereof, and obtained from an animal immunised with a protective antigen against the disease pathogen as described above; and

a myeloma cell;

10 fusing the B cell with the myeloma cell;
propagating a hybridoma formed thereby, and
harvesting the antibody produced by said hybridoma.

The monoclonal antibodies may form the basis of a passive treatment of the disease discussed above.

15 Preferably the antigen is a Fasciola hepatica antigen.

The monoclonal antibodies so formed may be selected from the group consisting of FY 4-7-12, FY 3-3-1, FY 3-3-2, FY 3-5, FY 4-7-6 and FY 1-6 as hereinafter described.

20 Having identified the antigen(s) molecular biology or chemical techniques, e.g. cloning techniques may be used to produce unlimited amounts of this antigen or alternatively synthetic peptides corresponding to different fragments of the identified antigens may be used as a means to produce a vaccine.

Accordingly in a preferred aspect of the present invention there is provided a method for preparing a synthetic antigenic polypeptide against a
25 disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species, which method includes

providing

a cDNA library, or genomic library derived from a sample of a
disease pathogen selected from Fasciola, Ostertagia and
30 Trichostrongylus species, and related species; and

a corresponding antibody probe including

at least one antibody against the respective disease pathogen
produced by a method including providing a biological sample

from an immune animal taken a short time after the immune animal has been challenged with a pathogen or pathogen extract selected from Fasciola, Ostertagia and Trichostrongylus species, and related species, or
5 a corresponding monoclonal antibody derived therefrom, or a polyclonal or monoclonal antibody generated after injection of the purified antigen;

generating synthetic polypeptides from the cDNA library or genomic library;

10 probing the synthetic polypeptides with the antibody probe; and isolating the synthetic antigenic polypeptide detected thereby.

Either cDNA or genomic libraries may be used. The cDNA or genomic libraries may be assembled into suitable expression vectors that will enable transcription and the subsequent expression of the clone of DNA, either in
15 prokaryotic hosts (e.g. bacteria) or eukaryotic hosts (e.g. mammalian cells). The probes for screening the libraries may preferably be selected from:

- (i) synthetic oligonucleotide probes based on the amino acid sequence of the antigen identified and purified as described above;
- (ii) PCR products generated from the synthetic oligonucleotide probes;
- 20 (iii) antibodies based on synthetic peptides derived from amino acid sequence data of the antigen identified;
- (iv) antibodies obtained from the culture medium produced as described above;
- (v) monoclonal or polyclonal antibodies produced against the antigens
25 identified and purified as described above; and
- (v) recombinant or synthetic monoclonal antibodies or polypeptides with specificity for the antigen, e.g. as described by Ward et al 1989, Nature 241, pages 544 to 546.

Preferably the cDNA library is derived from a sample of Ostertagia circumcincta; the corresponding antibody probe is a monoclonal antibody raised
30 against the 32-36 kD doublet antigen.

In a still further aspect there is provided a synthetic antigenic polypeptide prepared as described above.

The synthetic antigenic polypeptide may be selected from clones 3-2 and 5-2b, having the amino acid sequence

M.A.FETNYP IPYRSKLTEP FEPGQTLTVK GKTGEDSVRF TINLHNSSAD
FSGNDVPLHV SVRFDEGKIV CNSFAKGEWGKEERKSNPYK KGDDIDIRIR
5 AHDSKFQIFV DQKELKEYEH RLPLSSITHF SIDGDVLITH IHWGGKYYPV
PYESGLAGEG LSPGKSLYLY GMPEKKGKRF HINILKKNKD IALHFNPRFD
EKAIVVRNSLI SNEWGNEERE GKMPFEKAVG FDLEIKNEDY PFQIMVNGER
FASYSHRLEP HELNGLQIGG DVEITGIQLH

, as hereinafter described.

10 Accordingly in a further aspect of the present invention, there is provided a putative protective antigen against a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species, prepared by a method including

providing

15 a sample of a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species; and

an antibody probe including at least one antibody against a disease pathogen selected from Ostertagia and Trichostrongylus species, and related species, produced by a method as described above;

20 probing the disease pathogen sample with the corresponding antibody probe; and

isolating the protective antigen detected.

The protective antigens may function as vaccine and/or diagnostic antigens as discussed below.

25 In another aspect of the present invention, there is provided a monoclonal or polyclonal antibody against a protective antigen, or fragment thereof, against a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species.

In another aspect, there is provided a monoclonal or polyclonal antibody
30 against a protective antigen as hereinbefore described, or fragment thereof, against Fasciola species, and related species.

In another aspect, the present invention provides a method for preventing infection caused by a disease pathogen selected from Fasciola, Ostertagia and

Trichostrongylus species, and related species, in animals, which method includes administering to an animal an effective amount of at least one protective antigen as described above.

Preferably, the protective antigen is an antigen derived from Fasciola
5 hepatica or Trichostrongylus colubriformis, as herein described.

In a still further aspect of the present invention there is provided a method for the treatment of infection caused by a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species, in animals, which method includes administering to an animal a therapeutically
10 effective amount of a monoclonal or polyclonal antibody to a protective antigen as described above.

The present invention further provides a vaccine or veterinary composition including a prophylactically effective amount of at least one antigen against a disease pathogen selected from Fasciola, Ostertagia and
15 Trichostrongylus species, and related species, as described above. Preferably the vaccine composition includes a plurality of protective antigens against a number of disease pathogens.

The present invention further provides a vaccine or veterinary composition including a therapeutically effective amount of at least one
20 monoclonal or polyclonal antibody against a protective antigen as described above. Preferably the vaccine composition includes a plurality of monoclonal or polyclonal antibodies.

In the preferred forms, multiple protection may be provided to an animal via a single treatment.

25 The vaccine or veterinary compositions according to the present invention may be administered orally or may be administered parenterally (for example by intramuscular, subcutaneous, intradermal or intravenous injection).

The amount required will vary with the antigenicity of the active ingredient and need only be an amount sufficient to induce an immune response typical of
30 existing vaccines.

Reactive experimentation will easily establish the required amount. Typical initial doses of vaccine or veterinary compositions may be approximately 0.001-1 mg active ingredient/kg body weight. The dose rate may increase or

multiple doses may be used as needed to provide the desired level of protection.

The vaccine or veterinary composition according to the present invention may further include a veterinary acceptable carrier, diluent or excipient thereof. Preferably the active ingredient may be suspended or dissolved in a carrier. The carrier may be any solid or solvent that is non-toxic to the animal and compatible with the active ingredient. Suitable carriers include liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, and solid carriers, such as talc or sucrose. Adjuvants, such as Freund's adjuvant, complete or incomplete, or immunomodulators such as cytokines may be added to enhance the antigenicity of the antigen if desired. When used for administering via the bronchial tubes, the vaccine is suitably presented in the form of an aerosol.

The vaccine or veterinary composition according to the present invention may be incorporated into a live vector (eg. vaccinia virus, salmonella) or administered as DNA or RNA, as described in Tang et al., Nature 356: 152, 1992.

In a still further aspect of the present invention there is provided a diagnostic assay kit including a diagnostic antigen, or fragment thereof, against a disease pathogen identified and purified as described above.

The diagnostic kit may be utilised to detect infections in animals caused by a disease pathogen selected from O. circumcincta, F. hepatica, I. colubriformis or related parasites.

The diagnostic assay kit may be utilised in conjunction with a diagnostic assay. The diagnostic assay may include Western blot techniques. The diagnostic assay may be a diagnostic immunoassay. The immunoassay may be an immunoprecipitation assay, a radioimmunoassay, an enzyme linked immunoassay, an immunofluorescent assay or a chemiluminescent assay.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

IN THE FIGURES:

FIGURE 1a: *Ostertagia circumcincta*

SDS-PAGE (12.5% gel) and Western blot analysis of L3 larval extracts of *Ostertagia circumcincta* probed with lymph (1/1000

- 14 -

dilution) from experimentally immune Suffolk lambs. Two clusters of immunoreactive species of apparent M.W. 26-36 and 95-105 kD were detected. The same regions were also identified with culture supernatant of abomasal lymph nodes from challenged sheep.

5 FIGURES 1b-1e:

Reaction of clones with anti-doublet mAb or sheep serum by plaque immunoassay

10 IPTG filters lifted from plates of each clone were cut into pieces and reacted with antibody. Detection was with alkaline phosphatase conjugated anti mouse IgM+IgG or anti-sheep IgG. Filters were reacted with Fig. 1b: anti-doublet mAb, Fig. 1c: a negative control IgM mAb, Fig. 1d: sheep serum raised to purified doublet antigen, Fig. 1e: negative control sheep serum. Clones shown are 7-1, 7-2, 5-2b (2 isolates), 3-2, 8-2 (2 different dilutions) or negative control
15 plaques.

FIGURE 1f: Western blot of O.circumcincta L3 extract probed with antibodies affinity purified from clones

20 Samples of aqueous extracts from L3 larvae were electrophoresed on a 12.5% SDS-polyacrylamide gel, which was then electroblotted. The Western blot was reacted with affinity purified antibodies eluted from plaque immunoassays, with mAbs, with sheep sera or with abomasal lymph from a repeatedly infected, immune sheep, and reactions detected with alkaline phosphatase anti mouse IgM+IgG or anti-sheep IgG. Lane 1: negative control mAb; 2: anti-doublet
25 mAb; affinity purified antibodies eluted from 3: clone 7-1; 4: clone 7-2; 5 and 6: clone 5-2b (2 isolates); 7: clone 3-2; 8: clone 8-2; 9: negative control plaques; 10: sheep anti-doublet serum; 11: negative control serum; 12: lymph from immune sheep; M: pre-labelled molecular weight markers (Biorad). The position of the
30 doublet is arrowed.

FIGURE 1g: Nucleotide and predicted amino acid sequence of clones 3-2 and 5-2b.

Amino acids are shown in the 3 letter code.

FIGURE 1h: Alignment of *O.circumcincta* clones 3-2 and 5-2b predicted amino acid sequence with GBPs from *O.volvulus* and *C.elegans*.

Amino acid sequences for the GBPs were extracted from databases using ANGIS (*O.volvulus* from GenPep database, accession no. U04046_1; *C.elegans* from PIR database, accession no. S27798). Single letter codes for amino acids are shown.

FIGURE 1i: Demonstration that the doublet antigen is a lectin-like GBP.

O.circumcincta L3 larvae were extracted with buffer and samples were applied to an asialofetuin-Affigel 15 column. After washing, bound protein was eluted with 100mM lactose. Lanes: M: molecular weight markers; 1: L3 extract; 2: last flow through fraction from column; 3-7: lactose elution fractions. The position of the doublet antigen is arrowed. (12.5% SDS-PAGE, coomassie stained).

FIGURES 1j and 1k:

Expression of recombinant antigens in *E.coli*.

Figs. 1j and 1k. CTAB-solubilised inclusion bodies were electrophoresed on a 13% gel which was then electroblotted. Half of the Western blot was reacted with anti-doublet mAb and detected with alkaline phosphatase conjugated anti-mouse IgG+IgM (Fig. 1j) and the other half was reacted with sheep antiserum to purified doublet and detected with alkaline phosphatase conjugated anti-sheep IgG (Fig. 1k). Lane 1: clone 7-1; 2: clone 7-2; 3: clone 3-2; 4: clone 8-2; 5: clone 5-2b; 6: pMOSELOX control.

FIGURES 2a-2c: - *Ostertagia circumcincta*

Mean eggs per gram faeces (epg) of vaccinated (●—●) and control (o—o) sheep after infection with L3 larvae in three separate trials.

FIGURE 2d: Mean faecal egg counts of control and vaccinated sheep used in the third *Ostertagia* vaccination trial after heterologous infection with *H. contortus*.

FIGURE 3: *Trichostrongylus colubriformis*

Western blot of 3 species of L3 nematode larval antigens probed with MLN supernatant of *T.colubriformis* infected sheep.

Lane 1. Bio-Rad prestained molecular weight markers.

- 16 -

Lane 2. O. circumcincta L3 antigen.

Lane 3. H. contortus L3 antigen.

Lane 4. T. colubriformis L3 antigen.

Brackets = antigen of Trichostrongylus colubriformis

5 **FIGURE 4:** Fasciola hepatica

Western blot of NEJ fluke antigen probed with supernatant from hepatic lymph node (1), mesenteric lymph node (2), and spleen (3) 7 days after oral challenge with 200 Mc of previously infected and cured rats.

10 Lane 4: Bio-Rad Prestained molecular weight markers.

Arrow = position of >200 kD antigen recognised only by the MLN supernatant.

FIGURE 5: Fasciola hepatica

Western blot of NEJ antigen probed with supernatant from hepatic lymph node (5), mesenteric lymph node (6) and spleen (7) of twice immunised and cured rats challenged with 400 Mc.

15

Lane 4: Bio-Rad prestained molecular weight markers.

Arrows - position of antigens recognised only by the MLN supernatant.

FIGURES 6a-6d: - Fasciola hepatica

20

Indirect immunoperoxidase staining of NEJ flukes with (Fig. 6a) mcAb 38.27 against sheep MHC class II (negative control) X 40. Similar negative staining was observed with mcAb's FY3-5 and FY1-6, (Fig. 6b) mcAb FY3-3-2, X40; (Fig. 6c) mcAb FY3-3-2, X 100 and (Fig. 6d) mcAb FY3-3-1, X 100. Note the strong reticular type staining in (Fig. 6b) and (Fig. 6c) compared to the more restricted speckled staining in (Fig. 6d). The arrows point to the oral sucker of the NEJ flukes.

25

EXAMPLE 1

OSTERTAGIA CIRCUMCINCTA

30 **Parasites and Experimental Animals**

O. circumcincta third stage larvae (L3) were collected from faecal cultures of donor sheep experimentally infected with the parasite. Immune animals were obtained by repeatedly infecting sheep with O. circumcincta larvae and then

monitoring faecal egg output. When a challenge dose produced few or no eggs in the faeces the animal was said to be immune. Once immune, the sheep were drenched with IVERMECTIN and left for a period of at least four weeks before being challenged with 60,000 L3 larvae and then killed five to eight days post challenge.

Preparation of Culture Supernatants

Abomasal lymph nodes (ALN) were removed and cell suspension prepared as described in Australian Patent No. 640364 referred to above. Bulk cultures of 10-50 ml were set up in culture flasks (Miles) at a concentration of 0.5-
10 1.0×10^7 cells/ml in DME + 10% foetal calf serum. Preliminary experiments established that most of the antibodies in the culture supernatant were produced by the antibody secreting cells present in the *in vivo* stimulated lymph nodes and that this was not further increased by stimulation with pokeweed mitogen (PWM). PWM was therefore not added to further cultures and culture supernatants were
15 harvested after a five day incubation of cells at 37°C in 5% CO₂ atmosphere, then stored at -20°C until used.

Cannulation of abomasal lymph nodes and collection of lymph

Sheep (Suffolk lambs) were rendered immune as described above, challenged with 60,000 L3 larvae and the common abomasal lymph duct
20 cannulated 4 days after challenge. Lymph was collected for several days after cannulation and the cell-free lymph stored at -20°C prior to use.

Preparation of Antigens for SDS-PAGE and Western blots

Third stage larvae of *O. circumcincta* were exsheathed in approximately 0.5% NaHOCl in a CO₂ enriched atmosphere for 20 minutes at 37°C to remove
25 the second stage sheath. The larvae were then repeatedly washed and centrifuged at 3,000 g for 10 minutes in phosphate buffered saline (PBS) pH 7.4. After the sixth wash they were transferred to 500 ml of DME medium pH 6.8 in the presence of 200 U/ml penicillin and 0.2 µg/ml streptomycin and cultured at 39°C with 20% CO₂ in air for 3 days. The culture media was then centrifuged at 3,000
30 g for 15 minutes at 20°C and the pelleted in-vitro switched L4 larvae stored at -70°C.

Antigens were extracted from exsheathed L3, in-vitro switched L4, in-vivo

L4 and adult stages by freeze thawing 3 times, then homogenisation using a polytron homogeniser (Kinematica GmbH, Switzerland), extraction overnight in 50mM Tris HCl pH 8.0, 150mM NaCl, and centrifugation at 50,000 xg for 30 minutes. The supernatant containing solubilised antigens was stored at -70°C.

- 5 The extracted antigens were run under non-reducing conditions on 12.5% (W/V) SDS-polyacrylamide gels and western blotted onto PVDF membrane (Immobilon, Millipore) or nitrocellulose.

Preparation of antigens for vaccination trials

- Third stage larvae of *O. circumcincta* were exsheathed in CO₂ enriched
10 atmosphere for 2-3 hours at 37°C to remove the second stage sheath. The exsheathed L₃'s were freeze-thawed three times, then homogenised using a ground glass homogeniser followed by overnight extraction in 150mM NaCl, 50mM Tris pH 8.0 containing 2% (w/v) Hexadecyltrimethyl- ammonium bromide (CTAB, Sigma). Particulate matter was removed by 30 min centrifugation at
15 300g. The supernatant was further centrifuged at 15,000 xg for 30 min and the soluble extract stored at -20°C.

- The extracted antigens were run under non-reducing conditions (without boiling) on 10% CTAB-acrylamide gels. The appropriate area of gel was identified by western blotting 2 strips on either end of the gel and reacting the
20 strips with positive lymph. The gel area corresponding to the immuno-reactive region was excised, mashed and incubated in 2% CTAB solution overnight to passively elute the antigens. The CTAB was removed by incubation with Dowex resin in 2M urea at pH 10 for 15 min, then dialysed against PBS overnight to remove the urea. The antigen was concentrated in Centriprep concentrator
25 (Amicon), the protein concentration determined using BCA assay (Pierce) and used to immunise sheep. This antigen preparation was shown to contain the 26-36kD immunoreactive region when run on SDS-PAGE gels.

Identification of Antigen

- Western blotted antigen preparations were probed with in vitro culture
30 supernatant from ALN and lymph from cannulated abomasal lymph nodes from infected sheep. Both the culture supernatant and the lymph highlighted two regions of molecular weights 26-36 and 95-105 kD (see Figure 1).

Incubation of the blotted antigens with lectin conjugates revealed that the antigens were able to bind some lectins. This indicates that some of the antigens are glycosylated.

Protein Sequencing of the 26-36kD Antigens Identified by Western Blotting

5 The N-terminal amino acid sequences of the antigens within this region were determined after separating the proteins by SDS-PAGE, followed by electrophoretic transfer to ProBlott sequencing membrane using CAPS buffer. The transferred proteins were located on the membrane by staining with coomassie blue and were excised. The sequence was determined in an Applied
10 Biosystems 476A protein sequencer fitted with a blot cartridge. Further internal sequence data were generated by digesting the CTAB purified antigens with cyanogen bromide, separating the fragments by SDS PAGE, blotting onto ProBlot and sequencing as above. The results of the protein sequencing are summarised below. Two bands in the upper part of the 26-36kD region did not give any
15 sequence and were presumably blocked at the N-terminus. These two bands in the upper 32-36kD region are further referred to as "the doublet" since a monoclonal antibody generated against this region recognises both bands (see Fig. 1f) indicating that they are similar molecules.

20 The sequences obtained from the lower bands in the 26-36 kD region were screened against the sequence database libraries available at the Australian National Genomic Information Service, using the program FASTA. The results are shown below. Two of the molecules could be identified by virtue of their high degree of homology to sequences in the databanks. The homologous sequences were tropomyosin and glutathione S-transferase.

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25 The O. circumcincta sequences homologous to

(A) tropomyosin are:

1. N-terminal sequence: MKAE EVRQALK
2. Internal sequence : VEADLERAEERAE AAGENKVVL

(B) Glutathione S-transferase:

30 N-terminal: VQYKLYYFDGRXAAEV

To obtain sequence from the region containing the doublet bands, an aqueous extract of homogenised third stage Ostertagia circumcincta larvae was prepared and the proteins in the extract were separated by SDS PAGE under

- 20 -

reducing conditions. The doublet bands were excised from the coomassie blue stained gel and the proteins were then extracted from the excised gel pieces by electroelution. The isolated protein was digested with Trypsin and the peptides thus generated separated by high performance reversed phase liquid chromatography (HPLC). The purified peptides were sequenced by Edman degradation in an Applied Biosystems 476A protein sequencer. The following peptide sequences were determined

- 1) SAHGPPGQ
2) FPHGPSYQHGYA
3) IVTHPNR

Cloning of cDNAs encoding the doublet antigen

Library Preparation

RNA was extracted from freshly harvested stage L3 larvae, which had been snap-frozen in liquid nitrogen (Chomczynski & Sacchi, 1987, Anal.

Biochem., 162, 156-159). Messenger RNA (mRNA) was isolated by oligo dT

affinity chromatography on mAP paper (Amersham Australia). Double-stranded

complementary DNA (cDNA) was prepared by priming 2µg mRNA with either

oligo dT or random primers using a cDNA Synthesis System Plus kit (Amersham

Australia). Oligo dT and random-primed cDNA was pooled and EcoRI adaptors

were added (cDNA rapid adaptor ligation module, Amersham Australia), and the

adapted cDNA ligated to EcoRI cut, de-phosphorylated bacteriophage expression

vector λMOSELOX arms (Amersham Australia) and packaged using the λ-DNA in

vitro packaging module (Amersham Australia). A primary library of 1.4×10^6

plaque forming units (pfu)/mL, of which > 90% were recombinants, was obtained.

The library was amplified on E.coli ER1647 cells before use.

Library Screening

5×10^5 pfu of the amplified cDNA library were plated out on E.coli

BL21(DE3)pLysE cells at 5×10^4 pfu/plate. When pin-prick size plaques appeared

(after 4-6 hours at 37°C), the plates were overlaid with nitrocellulose filters which

had been impregnated with 10mM isopropylthio-β-galactoside (IPTG) and

incubated for a further 6 hours at 37°C. Plates were then stored overnight at 4°C.

Filters were removed from the plates and washed in TNT (10mM tris-HCl, pH 8,

150mM NaCl, 0.05% Tween 20), blocked in BLOTTO (5% w/v low fat milk powder

- 21 -

in TNT) and incubated for 2 hours at room temperature with an IgM mouse monoclonal antibody (mAb) (undiluted culture supernatant) which had been raised to the doublet antigen. After washing in TNT, filters were incubated with alkaline phosphatase conjugated rabbit anti-mouse IgG+IgM (Jackson Immunoresearch) at a 1:5,000 dilution in BLOTTO for 1 hour at room temperature. After further washing in TNT, filters were developed with 0.165mg/mL 5-bromo-3-chloro-4-indolyl phosphate (BCIP) and 0.33mg/mL nitroblue tetrazolium (NBT) in alkaline phosphatase buffer (0.1M tris-HCl, pH 9.5, 0.1M NaCl, 5mM MgCl₂). Fifteen putative positive plaques (some of which were very faint) were picked and re-screened with the monoclonal antibody as described above, after which 5 plaques remained positive. These plaques were tertiary screened and amplified stocks prepared by plating on ER1647 cells.

Analysis of clones

To determine the specificity of clones for the doublet antigen, plaque immunoassays were performed. Plaque-purified clones were plated out on BL21(DE3)pLysE *E. coli* cells and induced with IPTG filters as described above. The filters were then reacted with the anti-doublet mAb, an unrelated IgM mouse mAb, and with antiserum raised in sheep to purified doublet antigen or with negative control sheep serum (both sheep sera were used at a 1:50 dilution in BLOTTO, and the secondary antibody was alkaline phosphatase conjugated rabbit anti-sheep IgG from Jackson Immunoresearch at 1:5,000). Positive reactions were detected as described above. All clones were positive with the anti-doublet mAb and negative with the unrelated mAb and with negative sheep serum. Two clones, designated 3-2 and 5-2b, were strongly positive with the anti-doublet serum raised in sheep (Figures 1b-1e).

Clones, or λ MOSELOX control plaques, were plated out at 2,000 plaques per 80mM plate to achieve confluent lysis. As soon as pin prick plaques appeared, IPTG filters were added and the incubation continued overnight. Filters were washed extensively with TNT, blocked 1 hour in BLOTTO and incubated 4 hours at room temperature with the anti-doublet sheep antiserum (1:50 in BLOTTO). (Before use, the serum was depleted of anti-*E.coli* antibodies by incubation with filters from plates of wild-type λ MOSELOX plaques.) Filters were then washed 5 times in TNT, once in borate wash buffer (0.1M boric acid, 0.5M

- 22 -

NaCl, 0.05% Tween 20, pH 8) and once in PBS (140mM NaCl, 2.7mM KCl, 8mM Na₂HPO₄, 0.0015mM KH₂ PO₄). Bound, affinity-purified antibodies specific for each clone were eluted for 1 minute in 0.1M glycine, 0.15M NaCl, pH 2.6, 5 mL for each filter and immediately neutralised by adding to tubes containing 300µl of 1M tris-HCl, pH 8. The antibodies were dialysed against TNT for 1-2 hours at 4°C, low-fat milk powder added to 5% w/v and stored at -20°C. Aqueous extracts of L3 larvae were electrophoresed on a 12.5% denaturing SDS-polyacrylamide gel and proteins transferred to Immobilon membrane (Millipore) by electroblotting. The membrane was washed in TNT, blocked in BLOTTO and cut into strips. Strips were incubated with the affinity purified antibodies from the clones, with anti-doublet mAb or negative control mAb, or with sheep anti-doublet antiserum or negative control sheep serum at 4°C overnight. Detection was with alkaline phosphatase conjugated anti-species antibody followed by colour development with BCIP and NBT. Antibodies affinity purified on the 2 clones which were positive with sheep anti-doublet antiserum, 3-2 and 5-2b, specifically recognised the doublet band from the O.circumcincta larvae on the Western blot (Figure 1f).

The clones were rescued into the plasmid form (in pMOSELOX) by plating on E.coli BM25.8 cells in the presence of carbenicillin as recommended by the manufacturer of the vector (Amersham Australia). Plasmid DNA preparations were performed by alkaline lysis and CsCl density gradient as described in 'Molecular Cloning, A Laboratory Manual, second edition' (Sambrook, J., Fritsch, E.F. & Maniatis, T., 1989, Cold Spring Harbor Laboratory Press). Samples of plasmid DNA were digested with EcoRI and analysed on a 1% agarose gel in TAE buffer (40mM tris-acetate, pH 8, 1mM EDTA) containing 50µg/mL ethidium bromide. Clone 3-2 contained 3 EcoRI fragments of approximately 1000, 400 and 200 base pairs, while clone 5-2b contained 2 fragments of 1000 and 400 base pairs.

DNA Sequencing

DNA sequencing was performed by the dideoxy method using a Sequenase kit according to the manufacturer's instructions (United States Biochemical Corporation). Sequencing reactions were performed in the presence of α-³⁵S-dATP and gels autoradiographed. Initially, primers based on the vector

flanking the insert were used (T7 gene 10 and SP6 primers). Further primers to sequence the length of the inserts were designed based on the sequence obtained. Clones 3-2 and 5-2b contained identical DNA sequences, with the exception that clone 3-2 had a considerably longer 3' untranslated region, including the poly (A) tail of the mRNA. The DNA sequence and predicted amino acid sequence is shown in Figure 1g. The predicted amino acid sequence was used to search the combined protein databases using the BLAST program (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J., 1990, J. Mol. Biol. 215, 403-410) on the National Centre for Biotechnology Information computer, using ANGIS as an interface. The sequence was found to be highly homologous with a 32kD lectin-like β -galactoside-binding protein (GBP) from Caenorhabditis elegans and Onchocerca volvulus. The O.circumcincta sequence had a 69% identity with the C.elegans sequence and a 78% identity with the O.volvulus sequence. The alignment of the amino acid sequences is shown in Figure 1h. By analogy with these homologous sequences, both the clones contain the initiating ATG.

Proof that the doublet is a lectin-like β -galactoside-binding protein

Features that the doublet antigen and the C.elegans 32k GBP have in common include size on SDS-PAGE, a lack of glycosylation and a blocked N-terminus (see Hirabayashi, J., Satoh, M. and Kasai, K., 1992, J. Biol. Chem. 267, 15485-15490). A feature of the C.elegans GBP is that it can be affinity purified by binding to an asialofetuin column (Hirabayashi, J., Satoh, M., Ohyama, Y. & Kasai, K., 1992, J. Biochem. Tokyo 111, 553-555).

O.circumcincta L3 larvae were extracted in aqueous buffer (150mM NaCl, 2mM EDTA, 50mM tris-HCl, pH 8) and the extract was applied to an asialofetuin-Affigel 15 column. Bound proteins were eluted with the above buffer containing 100mM lactose. Analysis by SDS-PAGE revealed that the doublet bound to the asialofetuin column (Figure 1i). This demonstrates that the doublet is indeed a lectin-like β -galactoside-binding protein. As both bands of the doublet bind to the sugar column, they are both lectin-like GBPs. It is not known why this GBP appears as 2 bands in O.circumcincta as it is only a single band in C.elegans. Perhaps the O.circumcincta protein undergoes some degree of post-translational cleavage.

The function of these GBPs in nematodes is not known, but for C.elegans it has been postulated that they are involved in regulation of morphogenesis, for example in cuticle formation.

Expression of recombinant antigens

5 Plasmid DNA from the clones or pMOSELOX control were transformed into E.coli BL21(DE3)pLysE cells using the simple transformation method of D. Hanahan (In 'DNA Cloning: A Practical Approach', Volume 1, 1985 (ed. D.M. Glover), IRL Press, Oxford, p115). It was necessary to transfer the plasmids from BM25.8 to this strain because the recombinant antigens are expressed under
10 control of the T7 promoter, and BL21(DE3)pLysE cells carry the gene coding for T7 polymerase under control of the lac promoter. Colonies were picked and grown overnight at 37°C. 10mL cultures were inoculated at a 1:100 dilution from the overnight cultures and grown for 5 hours. Recombinant protein synthesis was induced by the addition of IPTG to 0.1mM and the incubation continued overnight.

15 E.coli cell pellets were resuspended in PBS containing 0.1% Triton X-100 and disrupted by sonication. Insoluble material, including inclusion bodies, was pelleted and resuspended by sonication in 1% CTAB. Samples were analysed by SDS-PAGE and by Western blotting (Figures 1j and 1k). Clones 3-2 and 5-2b produced fusion proteins which were positive on Western blots probed with the
20 anti-doublet mAb or with sheep antiserum to doublet antigen. In contrast, fusion proteins produced by clones 7-1, 7-2 and 8-2 were positive with the doublet mAb but not with the sheep antiserum, confirming the results of the plaque immunoassay described earlier (Figures 1b-1e). The pMOSELOX control protein did not react with either antibody. All the recombinant fusion proteins, and the
25 pMOSELOX control protein, were located exclusively in the CTAB-solubilised cell pellet, indicating that they were expressed in inclusion bodies.

Vaccination trials

Three vaccination trials were performed using CTAB extracts of native O. circumcincta antigens containing the 26-36kD immunoreactive region.

30 Vaccinated sheep were immunised 3 times, 2 to 3 weeks apart, with 50-100µg of protein in quil A for each immunisation. Control sheep received quil A only. All immunisations were given intradermally. All sheep were challenged with

20,000 L₃ larvae 2 to 3 weeks after the last immunisation and faecal egg counts monitored. Results of three separate vaccination trials are shown in Figures 2a-2c and show clear reduction in faecal egg counts in the vaccinated groups compared to the controls.

- 5 The first trial consisted of 5 vaccinated and 3 control sheep. The second trial had 10 vaccinated and 8 control sheep. The third trial consisted of 7 vaccinates and 7 control sheep.

Species cross-reactivity

- 10 A 26-36kD antigen region was also identified in an O. ostertagii antigen preparation when probed with sera from sheep vaccinated with O. circumcincta 26-36kD antigens (not shown). This indicates that similar antigens are also present in this and possibly other nematode species.

- 15 Peripheral blood lymphocyte proliferation assays of sheep at the end of the third Ostertagia vaccination trial were performed using a crude soluble H. contortus L3 extract. A highly significant ($P < 0.02$) stimulation with the H. contortus antigen was observed in the vaccinated sheep (mean 24003 cpm) compared to the control sheep (mean 6122 cpm) indicating cross reactivity between the two parasite species in the 26-36 kD antigen region. To assess crossprotection, all sheep of the third trial were drenched with ivermectin to
20 remove remaining Ostertagia worms and infected with 10 000 H. contortus larvae. As shown in Figure 2d, faecal egg counts were consistently lower in the vaccinated compared to the control group suggesting that cross protection has occurred. Because of the high variation in the faecal egg counts of the control group, no statistical significance was reached in the daily egg counts but there
25 was significant difference in the variance between the 2 groups (F-test). These two results show that there is significant heterologous stimulation and protection between Haemonchus and Ostertagia species and that similar protective molecules are likely to exist.

Characterisation of 95-105 kD antigen

- 30 Specific antisera prepared against the Haemonchus contortus 60-90 kD surface antigen described in Australian Patent 640,364 also reacted with O. circumcincta L3 larval extract in the same 95-105 kD region. This would suggest that this is a similar antigen to that described for H. contortus.

- 26 -

EXAMPLE 2TRICHOSTRONGYLUS COLUBRIFORMISExperimental Design

Sheep were immunised by several infections with T. colubriformis and left
5 uninfected for at least 4 months. They were challenged with 50,000 T. colubriformis L3 larvae, killed 10 days later and the first mesenteric lymph node (MLN) removed. Lymph node cells were processed and cultured as described for O. circumcincta and the supernatant was used to probe a Western blot of parasite antigens. L3 larval antigen extracts from O. circumcincta, H. contortus and T. colubriformis
10 were prepared as described previously for O. circumcincta.

The extracted antigens were run under reducing conditions on 12.5% (w/v) SDS-PAGE and Western blotted onto PVDF membrane (Immobilon, Millipore). The Western blot was probed with MLN-supernatant and developed with a peroxidase conjugated anti-sheep Ig (DAKO).

15 Results and discussion

A strong reaction was observed with the T. colubriformis antigen extract between the molecular weights of 32-35Kd and apparently consists of a doublet (Figure 3). No or a weaker reaction was observed with the O. circumcincta and H. contortus antigens. However, the T. colubriformis doublet antigen is in the
20 same position on SDS-PAGE as the O. circumcincta antigens described above and it is therefore possible that both are essentially similar molecules but with species-specific epitopes recognised most strongly by the homologous supernatant antibody probes.

EXAMPLE 3

25

FASCIOLA HEPATICA

We have used the technique of Western blotting with antibody probes obtained as described in Australian Patent No. 640364 to identify putative protective antigens of F. hepatica. A similar antigen may also be present in other Fasciola species (eg. F. gigantica) and in other trematode parasites (eg.
30 Schistosoma spp.).

Parasites and antigen extractions

F. hepatica metacercariae (Mc) were obtained from Ciba-Geigy (N.S.W., Australia). Newly excysted juveniles (NEJ) were obtained by in vitro excystation

as described previously (Australian Patent No. 640364). Juvenile liverflukes were recovered from mouse livers 17 days after oral infection. The different fluke stages were sonicated in PBS containing protease inhibitors, boiled in SDS non-reducing sample buffer, run on a 10% SDS-PAGE gel and transferred onto PVDF membrane (immobilon-P Millipore, MA) for Western blotting as described previously (Patent No. 640364).

Preparation of culture supernatant

In vitro cultures of spleen, hepatic lymph node (HLN) and mesenteric lymph node (MLN) cells were set up at 3×10^6 cells per ml in culture medium (DME containing 10% foetal calf serum, 2mM glutamine, 100u/ml penicillin, 100 µg/ml streptomycin and 2.5×10^{-5} 2-mercaptoethanol) essentially as described previously (Patent No. 640364). Supernatants were harvested after 4 to 5 days incubation and stored at -20°C until used.

Experimental design

8-9 week old PVC rats were infected with 100 *E. hepatica* Mc and treated 10 days later with the flukicide, Fasinex 120 (Ciba-Geigy) at 75 µg/gram. The rats were challenged orally 6 weeks later with 200 Mc and killed 7 to 10 days after challenge for collection of HLN, MLN and spleen cells.

Results

When no "breakthrough" infections had occurred (i.e. complete cure and elimination of the primary infection by the flukicide) there was a distinct difference in the local antibody response between the different lymphoid organs after a secondary challenge infection. No reaction was observed when spleen or HLN supernatants were used to probe a Western blot of NEJ antigen while a distinct antigen doublet was recognised by supernatant from the MLN cells. This antigen was located above the 110 kD molecular weight marker (Figure 4) and in later experiments (not shown) was seen to migrate above a 200 kD molecular weight marker and is further referred to as the >200 kD antigen.

When signs of "breakthrough" infections were detected (i.e. liver granulomas or adult flukes in bile ducts) a varied and complex pattern of NEJ antigen recognition was observed with HLN supernatant but the >200 kD antigen was again only and uniquely recognised by MLN cell supernatant after secondary

challenge infection (not shown).

All the rats were immune to the oral challenge infection as judged by the absence of macroscopically visible liver tracks and the total absence of juvenile flukes from mashed whole liver preparations.

- 5 When the same MLN supernatants as above were reacted on a Western blot with 17 day liverfluke antigen no such >200 kD doublet reaction could be observed (not shown) suggesting that this antigen is specific to the NEJ stage.

Conclusion

- 10 A doublet antigen of >200 kD molecular weight on a non-reducing SDS-PAGE gel has been discovered which is present in the NEJ stage that is uniquely recognised by culture supernatant of MLN cells during an early secondary response after oral challenge of immune rats. As the immunity developed in rats against oral challenge infection has been shown to occur at the level of the gut (reviewed in Vet. Parasitol. 1986, 20, 63-93) this antigen is a likely vaccine
- 15 candidate. The >200 kD antigen appears to be stage specific for the NEJ fluke as it was not detected under similar conditions in flukes collected from liver tissue and may therefore only be effective against the NEJ stage.

EXAMPLE 4

FASCIOLA HEPATICA

- 20 Parasites and antigen extractions along with the preparation of culture supernatant were carried out in the manner reported in Example 2.

Experimental Design

- 8-9 week old PVC rats were infected orally with 50 F. Hepatica Mc and cured 14 days later with 150 µg Triclabendazole/gram. They were given a
- 25 second oral infection of 150 Mc 3 days later and cured 4 days later as before. 1-2 months after the first infection rats were challenged orally with 400 Mc and killed 7 days later for collection of HLN, MLN and spleen cells.

Monoclonal Antibody (mcAb) Production

- Rats were immunised and challenged as above and killed 5 days after the
- 30 challenge infection. MLN cell suspensions were prepared and fused with a Y3 rat myeloma line provided by the MRC cellular immunology unit, Oxford, United Kingdom. Fusion supernatants were screened on Western blots against NEJ and 17 day liver stage antigen. Positive fusions were re-cloned out at least twice with

rat thymocytes as feeders.

Surface Staining with mcAbs

In vitro excysted NEJ's were incubated with mcAb supernatant containing 0.05% sodium azide for 30 minutes on ice. They were washed 3 times in cold
5 PBS-azide and incubated as before with a peroxidase conjugated rabbit anti-rat immunoglobulin (DAKO-Denmark) diluted 1/20 in PBS. After 3 washes in PBS colour development using Diamino benzidine substrate was allowed to proceed for a few minutes and stopped by dilution. After 2 more washes in PBS, the NEJ were fixed in PBS containing 1% formaldehyde and 2% glucose and staining
10 evaluated under a light microscope.

Results

The >200 kD antigen described in the conclusion of Example 3 was also present in the MLN supernatant of rats immunised twice. In addition this hyper-immune MLN supernatant also recognised an antigen of approximately 32 kD and
15 a diffuse antigen(s) with approximate molecular weight between 42 and 100 kD (Figure 5). The 2 extra antigens were also only detected on blots of NEJ antigen and not on 17 day liverstage antigen.

Monoclonal Antibodies (mcAb)

Several monoclonal antibodies were obtained from the fusion of twice
20 immunised and challenged MLN cells. Three of these mcAb's (F.h. 1-3) seemed to recognise the 3 antigens detected with the MLN supernatants. One mcAb was generated from the hepatic lymph node of infected rats (FY1-6) and recognised an antigen that was also strongly recognised with infection serum on both NEJ and liverstage fluke. All the mcAb's generated and their respective molecular
25 weight antigens are summarised in Table 1.

Later experiments (not shown) have established that the mcAbs FY4-7-12, FY3-3-1 and FY3-3-2 still react with peritoneal fluke stages collected 2 days but not 4 days after infection of naive mice.

Claims

1. A putative protective antigen or fragment thereof against Ostertagia circumcincta, or related infections, selected from antigens having approximate molecular weights in the region of 26-36 and 95-105 kilodaltons, as hereinbefore
5 described.
2. A putative protective antigen according to Claim 1 wherein the 26-36 kD antigen includes a doublet antigen in the 32-36kD position.
3. A putative protective antigen according to claim 2 wherein the doublet antigen is a lectin-like beta-galactoside-binding protein and may include one or
10 more of the peptide sequences
SAHGPPGQ
FPHGPSYQHGYA
IVTHPNR.
4. A putative protective antigen according to claim 1 wherein the antigen in
15 the 26-36 kD region is the O. circumcincta homologue of tropomyosin containing the following N-terminal sequence: MKAEEVRQALK and internal sequence: VEADLERAEERAEAAAGENKVVL.
5. A putative protective antigen according to claim 1 wherein the antigen in the 26-36kD region is the O. circumcincta homologue of glutathione S-transferase
20 containing the N-terminal sequence: VQYKLYYFDGRXAAEV.
6. A putative protective antigen, or fragment thereof, against Trichostrongylus colubriformis, or related infections, having an approximate molecular weight of 32-35 kilodaltons, as hereinbefore described.
7. A putative protective antigen according to claim 6 wherein the antigen is a
25 doublet antigen.
8. A putative protective antigen or fragment thereof against Fasciola hepatica or related infections selected from antigens having approximate molecular weights in the region of 28 kilodaltons, 32 kilodaltons, 37 kilodaltons, 42 to 100 kilodaltons, 54 to 55 kilodaltons and >200 kilodaltons, as hereinbefore
30 described.
9. A putative protective antigen according to claim 8 wherein the >200 kilodalton antigen is a doublet antigen.

10. A putative protective antigen according to claim 8 wherein the 32 kD antigen includes an N-terminal peptide sequence KPNYKRQFEPFSDELIHYINLE.
11. A putative protective antigen according to claim 8 wherein the 54-55 kD antigen includes an N-terminal peptide sequence LEDNGRTHWAVLVA.
- 5 12. A putative protective antigen according to Claim 8, wherein the Fasciola hepatica antigens are uniquely recognised by supernatant from mesenteric lymph node (MLN) cells in immune, challenged rats.
13. A method for preparing an antigen associated with a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related
- 10 species, according to any one of claims 1 to 12, which method includes providing
- a sample of a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species; and
- a corresponding antibody probe including at least one antibody
- 15 against the respective disease pathogen produced by a method including providing a biological sample from an immune animal taken a short time after the immune animal has been challenged with a pathogen or pathogen extract selected from Fasciola, Ostertagia and Trichostrongylus species, and related species;
- 20 isolating cells from the biological sample;
- culturing cells in vitro in a suitable culture medium; and
- harvesting antibodies produced from said cells,
- probing the pathogen sample to detect at least one antigen with the corresponding antibody probe; and
- 25 isolating the antigen detected.
14. A method according to Claim 13, wherein the sample of the disease pathogen is taken at a stage of development during which it is most susceptible to attack.
15. A method according to Claim 14, wherein the sample is taken from the
- 30 larval stage.
16. A method according to Claim 13, wherein the corresponding antibody probe is in the form of the supernatant harvested from the culture medium.

- 34 -

17. A method according to Claim 16, wherein the supernatant is from mesenteric lymph node (MLN) cells in immune challenged rats.

18. A method for producing a monoclonal antibody against an antigen of a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species according to any one of Claims 1 to 12, which method includes
5 providing

a B cell capable of producing antibodies against said protective antigen, or fragments thereof, and obtained from an animal immunised with a protective antigen against the disease pathogen as described
10 above; and

a myeloma cell;
fusing the B cell with the myeloma cell;
propagating a hybridoma formed thereby, and
harvesting the antibody produced by said hybridoma.

15 19. A method according to claim 18 wherein the antigen is a Fasciola hepatica antigen.

20. A monoclonal antibody against a protective antigen produced by a method according to Claim 18 or 19.

21. A monoclonal antibody against a Fasciola hepatica antigen selected from
20 the group consisting of FY 4-7-12, FY 3-3-1, FY 3-3-2, FY 3-5, FY 4-7-6 and FY 1-6 as hereinbefore described.

22. A method for preparing a synthetic antigenic polypeptide against a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species according to any one of Claims 1 to 12, which
25 method includes

providing

a cDNA library, or genomic library derived from a sample of a disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus species, and related species; and

30 a corresponding antibody probe including

at least one antibody against the respective disease pathogen produced by a method including providing a biological sample from an immune animal taken a short time after the immune

animal has been challenged with a pathogen or pathogen extract selected from Fasciola, Ostertagia and Trichostrongylus species, and related species, or a corresponding monoclonal antibody derived therefrom, or
5 a polyclonal or monoclonal antibody generated after injection of the purified antigen;

generating synthetic polypeptides from the cDNA library or genomic library;

probing the synthetic polypeptides with the antibody probe; and
10 isolating the synthetic antigenic polypeptide detected thereby.

23 A method according to Claim 22 wherein the cDNA library is derived from a sample of Ostertagia circumcincta; and the corresponding antibody probe is a monoclonal antibody raised against the 32-36 kD doublet antigen.

15 24. A synthetic antigen polypeptide produced by a method according to Claim 22 or 23.

25. A synthetic antigenic polypeptide, clones 3-2 and 5-2b having the amino acid sequence

M.A.FETNYP IPYRSKLTEP FEPGQTLTVK GKTGEDSVRF TINLHNSSAD
20 FSGNDVPLHV SVRFDEGKIV CNSFAKGEWGKEERKSNPYK KGDDIDIRIR
AHDSKFQIFV DQKELKEYEH RLPLSSITHF SIDGDVLITH IHWGGKYYPV
PYESGLAGEG LSPGKSLYLY GMPEKKGKRF HINILKKNKD IALHFNPRFD
EKAVVRNSLI SNEWGNEERE GKMPFEKAVG FDLEIKNEDY PFQIMVNGER
FASYSHRLEP HELNGLQIGG DVEITGIQLH

25 , as hereinafter described.

26. A diagnostic kit including a diagnostic antigen or fragment thereof according to any one of Claims 1 to 12.

27. A method for preventing diseases in animals, which method includes administering to the animal a prophylactically effective amount of at least one
30 protective antigen according to any one of Claims 1 to 12.

28. A method for the treatment of diseases in animals, which method includes administering to the animal a therapeutically effective amount of a monoclonal antibody to a protective antigen according to Claim 20 or 21.

- 36 -

29. A vaccine or veterinary composition including a prophylactically effective amount of at least one protective antigen against at least one disease pathogen selected from Fasciola, Ostertagia and Trichostrongylus according to any one of Claims 1 to 12.

5 30. A vaccine or veterinary composition according to Claim 29 including a plurality of protective antigens against a number of disease pathogens.

31. A vaccine or veterinary composition including a therapeutically effective amount of at least one monoclonal antibody according to Claim 20 or 21.

32 A vaccine or veterinary composition according to Claim 31 including a
10 plurality of monoclonal antibodies.

33. A putative protective antigen, or monoclonal antibody, substantially as hereinbefore described with reference to any one of the examples.

1/13

Fig.1a.

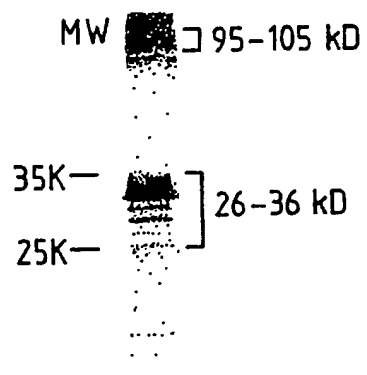
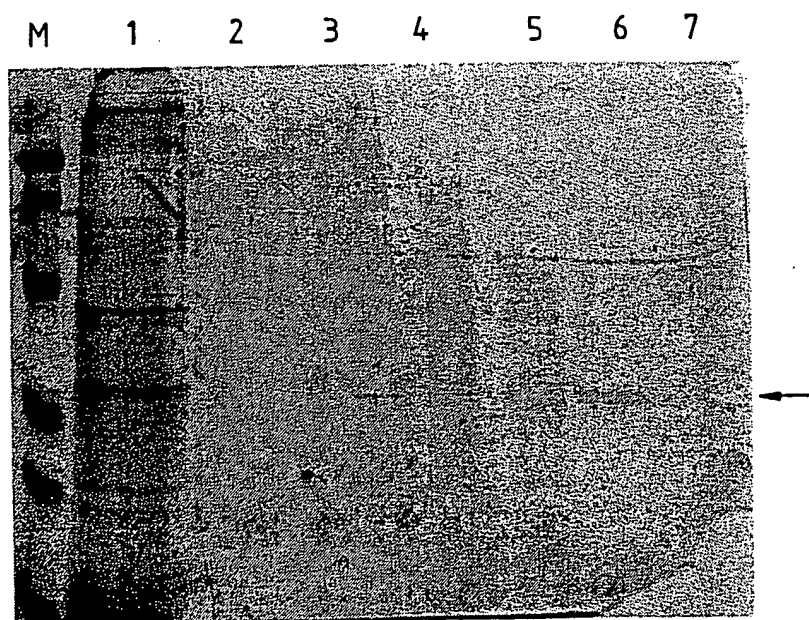


Fig.1i.



2/13

Fig.1b.

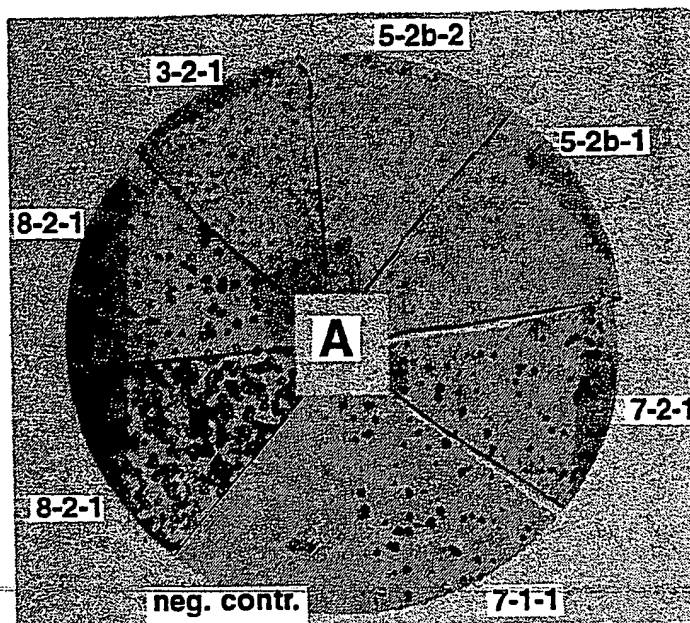
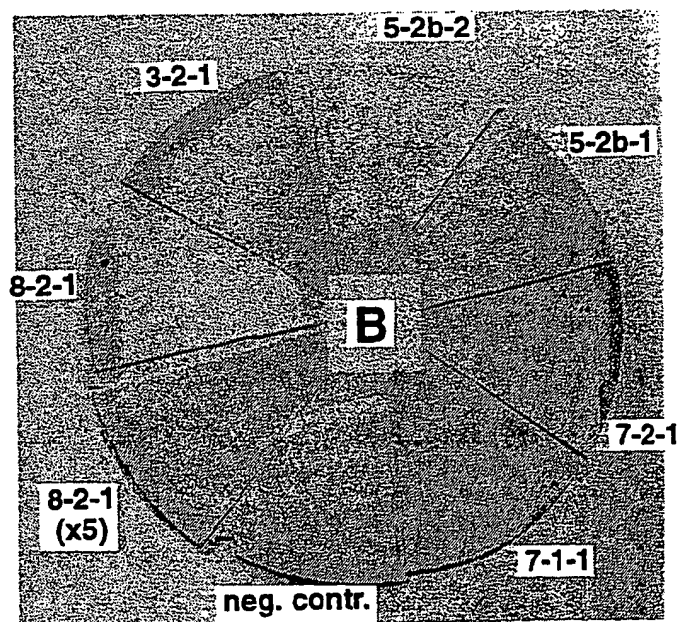


Fig.1c.



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3/13

Fig.1d.

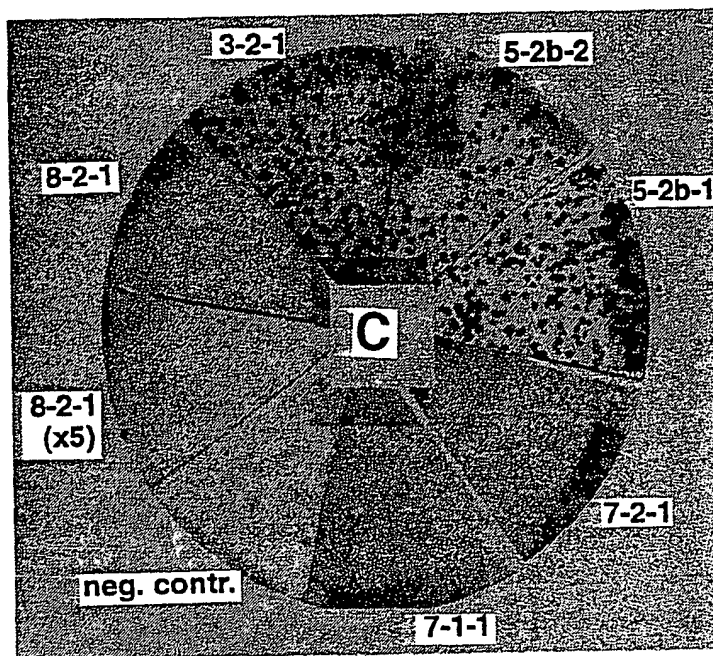
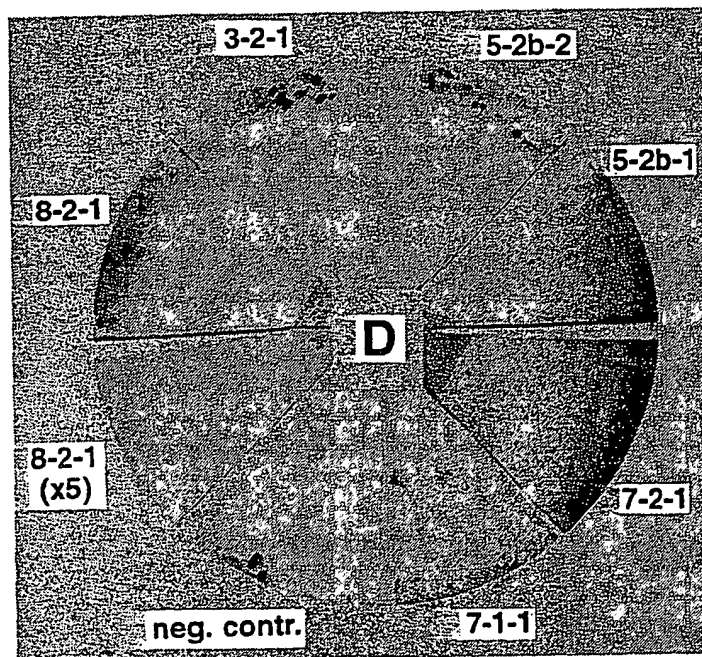
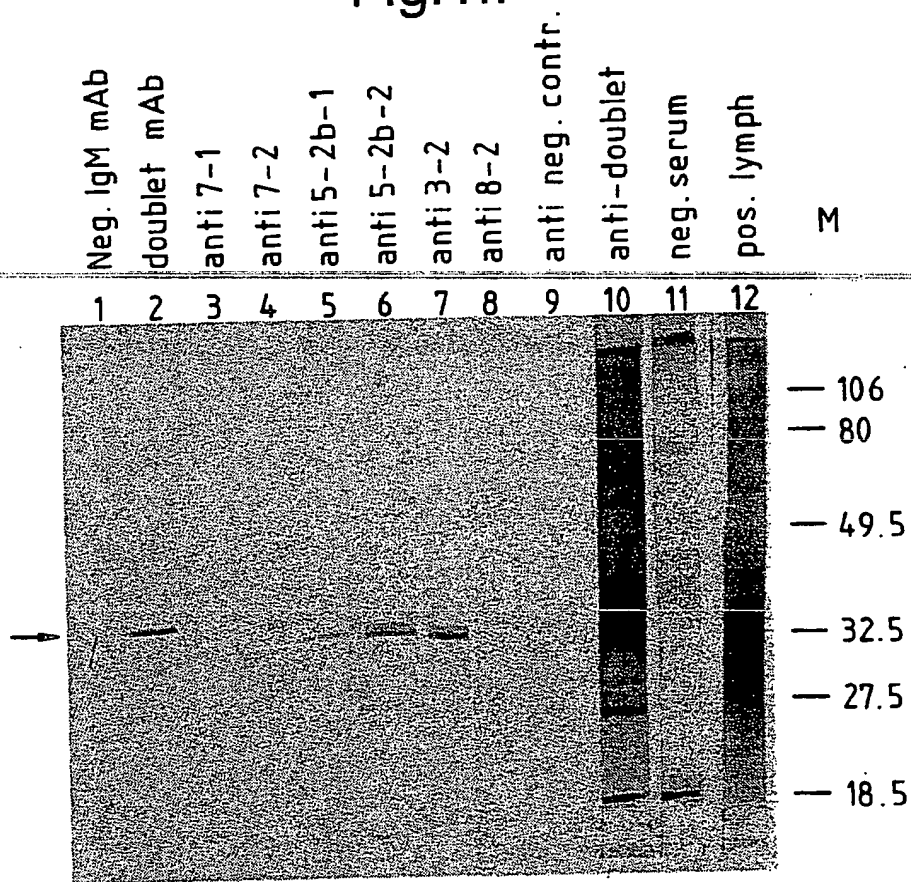


Fig.1e.



4/13

Fig.1f.



5/13

Fig.1g. Nucleotide and Predicted Amino Acid Sequence of the Coding Region of Doublet Clones 5-2b and 3-2

ATG GCT TTC GAA ACC AAC TAT CCG ATT CCA TAC CGA AGC AAA CTG ACC GAG CCA TTC GAA	30	60
Met Ala Phe Glu Thr Asn Tyr Pro Ile Pro Tyr Arg Ser Lys Leu Thr Glu Pro Phe Glu		
CCT GGA CAG ACA CTT ACT GTG AAG GGA AAG ACT GGC GAG GAC TCC GTC CGT TTC ACC ATC	90	120
Pro Gly Gln Thr Leu Thr Val Lys Gly Lys Thr Gly Glu Asp Ser Val Arg Phe Thr Ile		
AAC CTG CAC AAC TCG GCT GAC TTC TCC GGA AAC GAT GTT CCC CTT CAT GTC AGT GTG	150	180
Asn Leu His Asn Ser Ser Ala Asp Phe Ser Gly Asn Asp Val Pro Leu His Val Ser Val		
CGT TTC GAC GAA GGA AAG ATC GTC TGT AAT TCA TTT GCA AAG GGA GAA TGG GGA AAG GAA	210	240
Arg Phe Asp Glu Gly Lys Ile Val Cys Asn Ser Phe Ala Lys Gly Glu Trp Gly Lys Glu		
GAG AGA AAG AGC AAT CCA TAC AAG AAA GGA GAC GAC ATC GAC ATC CGT ATC CGA GCT CAC	270	300
Glu Arg Lys Ser Asn Pro Tyr Lys Lys Gly Asp Ile Asp Ile Arg Ile Arg Ala His		
GAC AGC AAA TTC CAG ATC TTT GTT GAC CAG AAG GAA CTG AAA GAA TAC GAG CAC CGT CTC	330	360
Asp Ser Lys Lys Phe Gln Ile Phe Val Asp Gln Lys Glu Leu Lys Glu Tyr Glu His Arg Leu		
CCG CTC TCA TCC ATC ACC CAT TTC TCA ATT GAC GGC GAT GTT CTC ATT ACA CAT ATC CAT	390	420
Pro Leu Ser Ser Ile Thr His Phe Ser Ile Asp Gly Asp Val Leu Ile Thr His Ile His		

6/13

Fig.1g(Cont).

480	TGG GGA GGA AAA TAC TAC CCT GTT CCA TAC GAG AGT GGA CTG GCT GGA GAA GGC CTT TCT	450	Trp Gly Gly Lys Tyr Tyr Pro Val Pro Tyr Glu Ser Gly Leu Ala Gly Glu Gly Leu Ser
540	CCA GGC AAA AGC CTT TAT TTG TAC GGA ATG CCG GAG AAG AAA GGA AAG CGA TTC CAC ATC	510	Pro Gly Lys Ser Leu Tyr Tyr Leu Tyr Gly Met Pro Glu Lys Lys Gly Lys Arg Phe His Ile
600	AAC ATT CTT AAG AAG AAC AAC GGC GAC ATC GCT CTG CAT TTC AAC CCG AGA TTC GAC GAG AAG	570	Asn Ile Leu Lys Lys Asn Gly Asp Ile Ala Leu His Phe Asn Pro Arg Phe Asp Glu Lys
660	GCT GTG GTA CGC AAC TCA TTG ATC AGC AAC GAA TGG GGT AAT GAA GAA CGC GAG GGC AAA	630	Ala Val Val Arg Asn Ser Leu Ile Ser Asn Glu Trp Gly Asn Glu Glu Arg Glu Gly Lys
720	ATG CCT TTC GAG AAG GCA GTC TTC GAT CTC GAG ATC AAG AAC GAA GAC TAT CCC TTC	690	Met Pro Phe Lys Ala Ala Val Gly Phe Asp Leu Glu Ile Lys Asn Glu Asp Tyr Pro Phe
780	CAG ATC ATG GTG AAC GGC GAA CGA TTC GCT TCG TAC TCT CAT CGA CTC GAA CCA CAT GAG	750	Gln Ile Met Val Asn Gly Glu Arg Phe Ala Ser Tyr Ser His Arg Leu Glu Pro His Glu
837	CTG AAC GGG CTC CAG ATT GGT GGT GAT GTC GAG ATT ACC GGA ATT CAA CTC CAT TAA	810	Leu Asn Gly Leu Gln Ile Gly Gly Asp Val Glu Ile Thr Gly Ile Gln Leu His *

7/13

Alignment of *O. circumcincta* clones 3-2 and 5-2b predicted
amino acid sequence with GBPs

Fig. 1h.

	1				50
<i>O. circumcincta</i>	M.A.FETNYP	IPYRSKLTEP	FEPGQTLTVK	GKTGEDSVRF	TINLHNSSAD
<i>O. volvulus</i>	MTNEYETNYP	VPYRSKLTES	FEPGQTLTVK	GKTAEDSVRF	TINLHNSSAD
<i>C. elegans</i>	MSAEEPKSYP	VPYRSVLQEK	FEPGQTLTVK	GSTIDESQRF	TINLHSKTAD
	51				100
<i>O. circumcincta</i>	FSGNDVPLHV	SVRFDEGKIV	CNSFAKGEWG	KEERKSNPYK	KGDDIDIRIR
<i>O. volvulus</i>	FSGNDVPLHI	SVRFDEGKIV	FNTFSKGEWG	KEERKSNPYK	KGDDIDIRIR
<i>C. elegans</i>	FSGNDVPLHV	SVRFDEGKIV	LNSFSNGEWG	KEERKSNPIK	KGDSFDIRIR
	101				150
<i>O. circumcincta</i>	AHDSKFQIFV	DQKELKEYEH	RLPLSSITHF	SIDGDVLITH	IHWGGKYYPV
<i>O. volvulus</i>	AHDSKYTIYV	DQKEVKYEH	RVPLSAVTHF	SIDGDVLVTV	IHWGGKYYPV
<i>C. elegans</i>	AHDDRFQIIV	DHKEFKDYEH	RLPLSSISHL	SIDGDLVLNH	VHWGGKYYPV
	151				200
<i>O. circumcincta</i>	PYESGLAGEG	LSPGKSLYLY	GMPEKKGKRF	HINILKKNGD	IALHFNPRFD
<i>O. volvulus</i>	PYESGLSGEG	LVPGKSLLIF	ATPEKKGKRF	HINLLKKNGD	IALHFNPRFD
<i>C. elegans</i>	PYESGLA.NG	LPVGKSLLVF	GTVEKKAKRF	HVNLLRKNGD	ISFHFNPRFD
	201				250
<i>O. circumcincta</i>	EKAIVRNLSI	SNEWGNEERE	GKMPFEKAVG	FDLEIKNEDY	PFQIMVNGER
<i>O. volvulus</i>	EKAIVRNLSI	AGEWNGEERE	GKMLEKGIG	FDLEIKNEEY	AFQIFINGER
<i>C. elegans</i>	EKHVIRNSLA	ANEWNGEERE	GKNPFEKGVG	FDLVIQNEEY	AFQVVFVNGER
	251				280
<i>O. circumcincta</i>	FASYSHRLEP	HEINGLOIGG	DVEITGIQLH		
<i>O. volvulus</i>	YATYAHRLDP	REINGLOIGG	DLEVSGIQMH		
<i>C. elegans</i>	YISFAHRADP	HDIAGLQISG	DIELSGIQIQ		

8/13

Fig.1j.

A

1 2 3 4 5 6

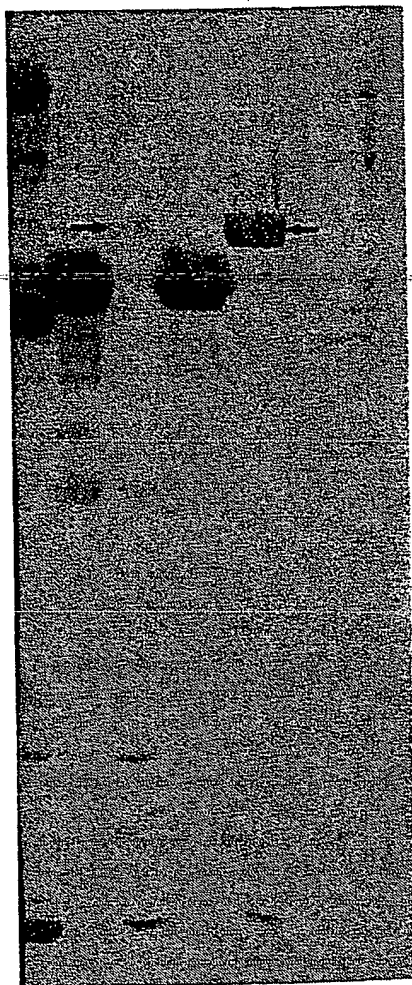


Fig.1k.

B

1 2 3 4 5 6

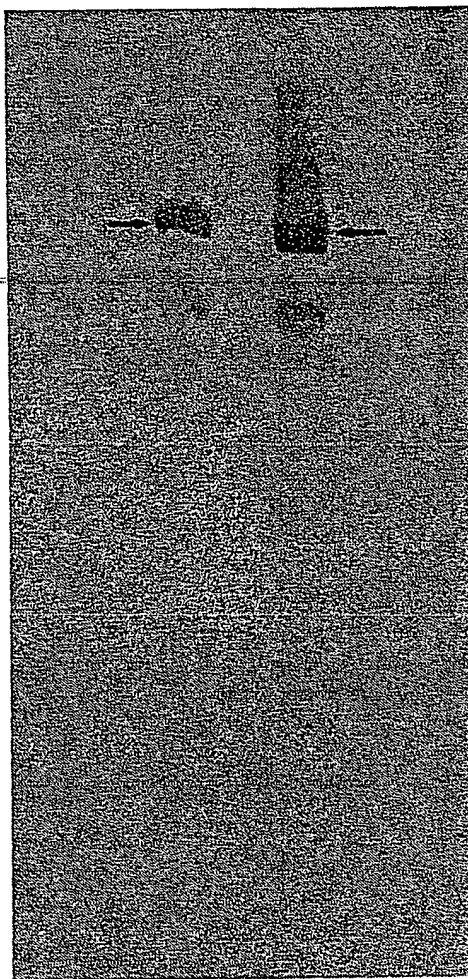


Fig.2a.

Trial 1 Faecal Egg Counts

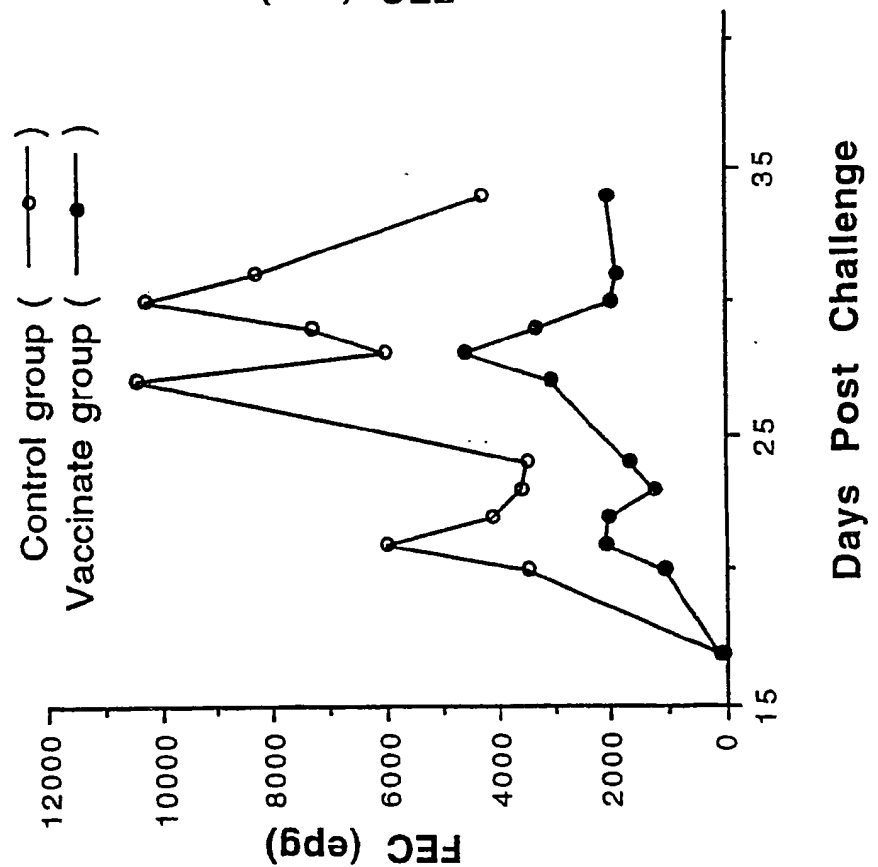


Fig.2b.

Trial 2 Faecal Egg Counts

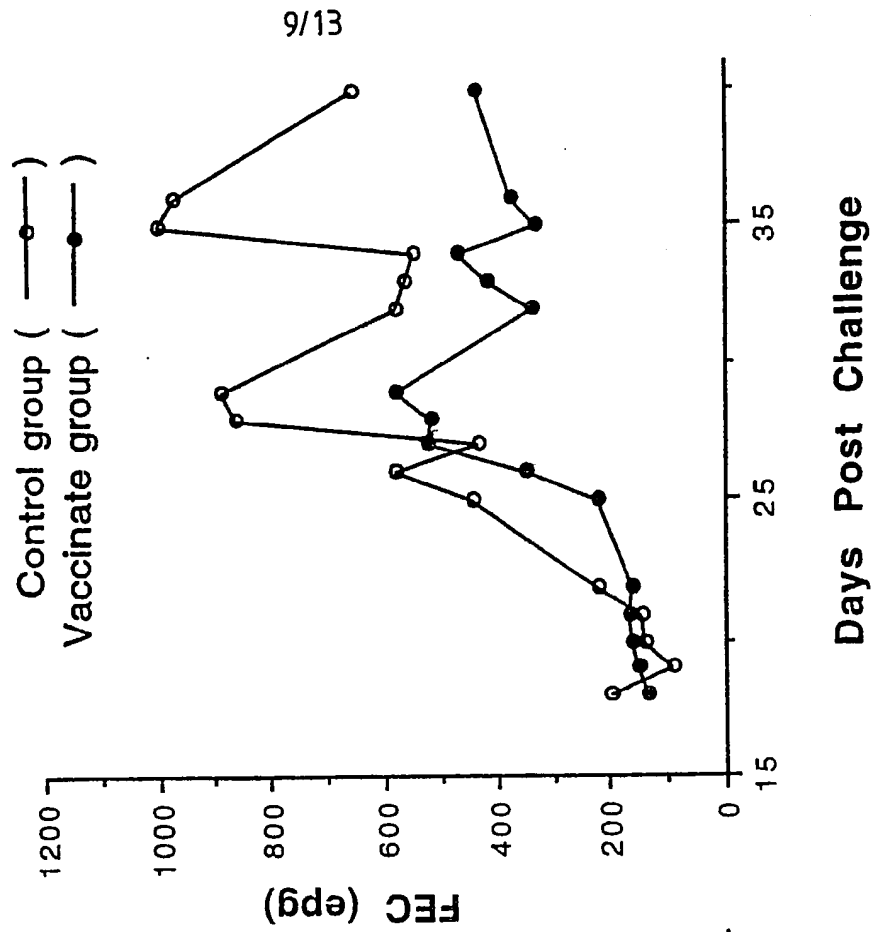
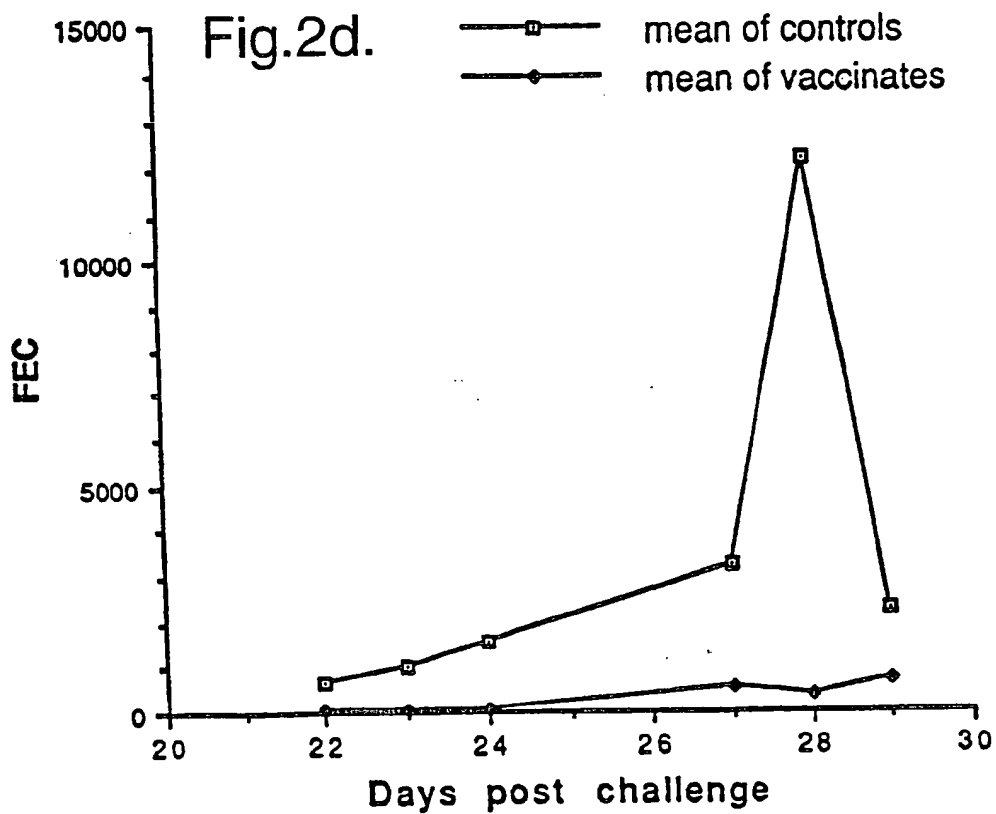
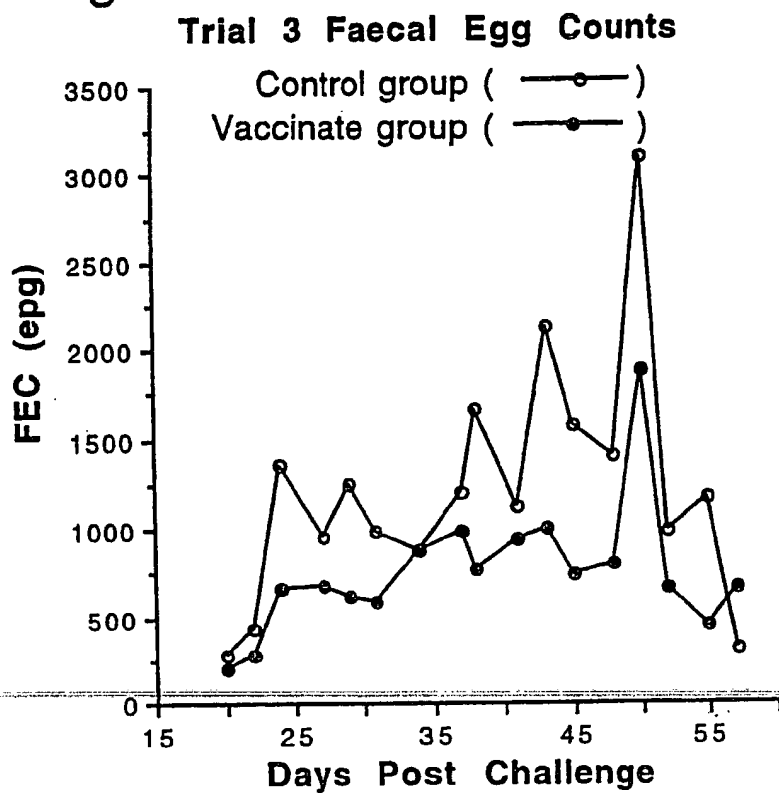
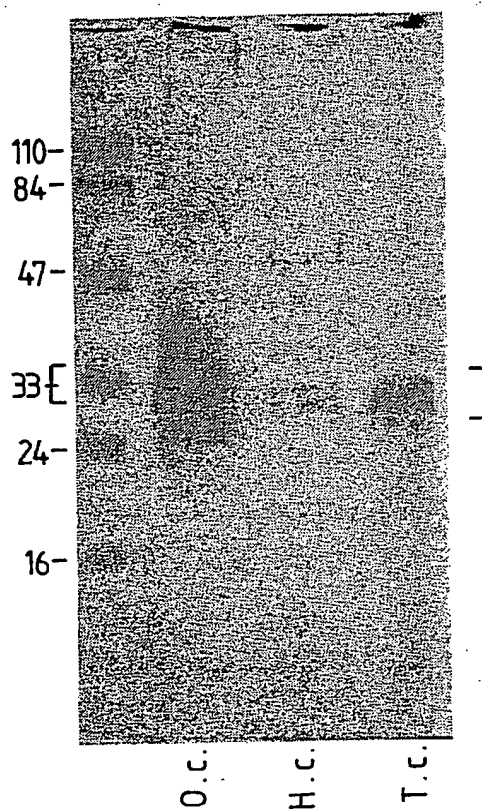


Fig.2c. 10/13



11/13

Fig.3.



12/13

Fig.4.

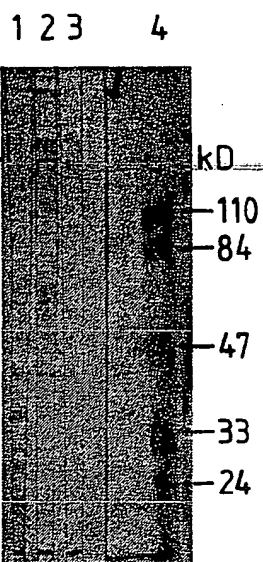


Fig.5.

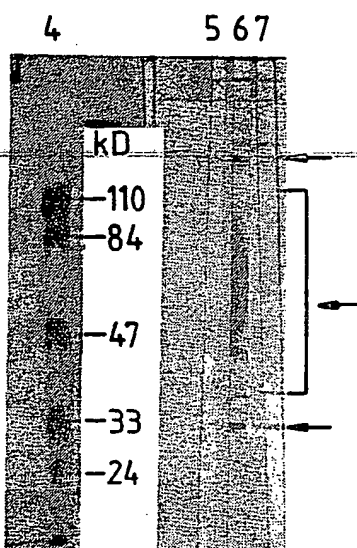


Fig.6a.

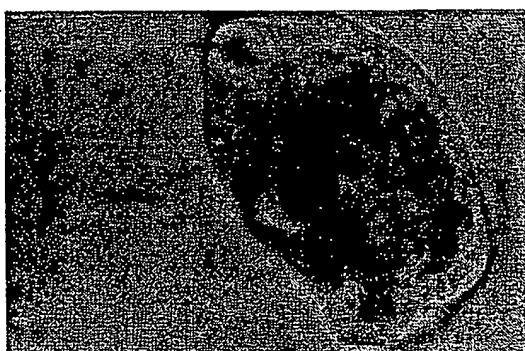


Fig.6b.

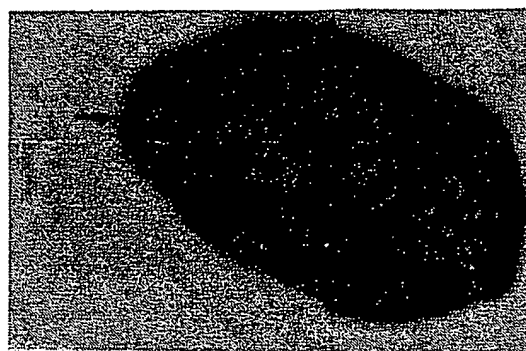


Fig.6c.

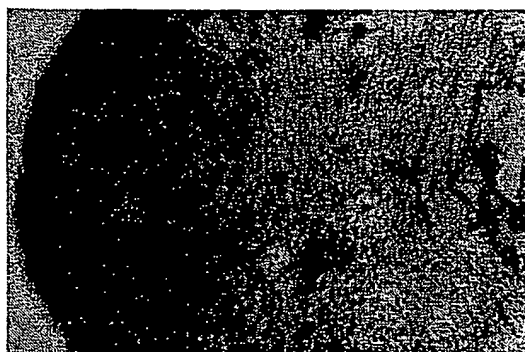
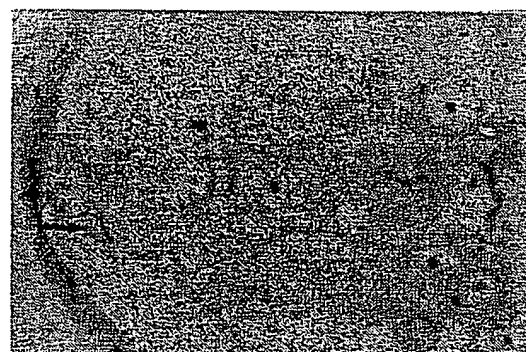



Fig.6d.



A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁶ C07K 14/435, 16/18, C12N 15/12, A61K 37/02, 39/00, 39/395 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC C07K Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC as above Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT CHEMICAL ABSTRACTS STN protein sequence search		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Vaccine, Volume 10, No. 9, 1992, D J McGillivray et al., "A purified stage-specific 31kDa antigen as a protective antigen against <u>Ostertagia circumcincta</u> infection in lambs", pages 607-613 entire document	1, 18, 20, 22, 24, 26-32
Y	entire document	13-17
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
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Date of the actual completion of the international search 2 December 1994 (02.12.94)		Date of mailing of the international search report 20 Dec 1994 (20.12.94)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  T. SUMMERS Telephone No. (06) 2832291

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X Y	Int J Parasitol, 1989, 19(5), 473-8 McGillivray et al., "Extraction and identification of a 31000 mol. wt glycoprotein antigen of <u>Ostertagia circumcincta</u> by sera from resistant sheep" entire document entire document	1, 18, 20, 22, 24, 26-32 13-17
X Y	Mol. Biochem Parasitol, 1990, 41(2) 167-76, Keith W Savin et al., "Characterization, cloning and host-protective activity of a 30-kilodalton glycoprotein secreted by the parasitic stages of <u>Trichostrongylus colubiformis</u> " entire document entire document	6, 18, 20, 22, 24, 26-32 13-17
X Y	Vet Parasitol, 1991, 40, 227-39, M Solano et al., "Production and characterization of monoclonal antibodies against excretory-secretory products of <u>Fasciola hepatica</u> " entire document entire document	8, 18-20, 22, 24, 26-32 13-17
X Y	Mol. Biochem Parasitol, 1992, 55, 155-65, M Soledad Marin et al., "Identification and expression of a <u>Fasciola hepatica</u> gene encoding a gut antigen protein bearing repetitive sequences" entire document entire document	8, 18-20, 22, 24, 26-32 13-17
P,X P,Y	Chemical Abstracts, Volume 121, No. 13, issued 26 September 1994 (26.09.94), M A Ruiz-Navarrek et al., "Fasciola hepatica: characterization of somatic and excretory-secretory antigens of adult flukes recognized by infected sheep", Abstract No. 155127n entire abstract entire abstract	8, 18-20, 22, 24, 26-32 13-17
A	J Clin Microbiol, 1990, 28(12) 2637-40, Ana M Espino et al. "Detection of circulating excretory secretory antigens in human fascioliasis by sandwich enzyme-linked immunosorbent assay" entire document	8, 18-20, 22, 24, 26-32
A	Vet. Parasitol, 1992, 42(1-2) 53-66, Didier Levieux et al., "An improved passive hemagglutination test for the serological diagnosis of bovine fascioliasis using the specific antigen f2" entire document	8, 18-20, 22, 24, 26-32
X Y	WO,A, 92/03735 (BRITISH TECHNOLOGY GROUP PLC) 5 March 1992 (05.03.92) abstract page 4 lines 29-34 abstract, page 4 lines 29-34	8, 18-20, 22, 24, 26-32 13-17
X Y	WO,A, 86/03680 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 3 July 1986 (03.07.86) abstract, page 13 line 20-page 14 line 20 and page 18 lines 19-25 abstract, page 13 line 20-page 14 line 20 and page 18 lines 19-25	8, 18-20, 22, 24, 26-32 13-17

PCT/AU 94/00573

SDOCID: <WO__9509182A1_I_>

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WO	9203735	CA	2089157	GB	2249097	AU	84265/91
WO	8603680	AU	53102/86	EP	211001	GB	2169606
		NZ	214652	US	4743446		
WO	9417820	AU	59962/94	CA	2126455		
AU	50283/90	BR	9007070	CA	2045663	WO	9008819
		EP	456662	NO	912942	NZ	232327
AU	49035/90	AP	144	BR	9000451	CA	2008808
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